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Loss of the retinoblastoma tumor suppressor protein (pRb) can contribute to breast tumor formation. However, many breast tumors retain expression of normal pRb, indicating that other genetic events may interfere with the function of this growth regulatory protein. Overexpression of cyclin D1 is such an event, since high levels of this protein may lead to constitutive inactivation of pRb through phosphorylation. We have demonstrated that cyclin D1 in oncogenic in cultured cells, and are using this assay to define regions and functions of cyclin D1 needed for transformation. Interestingly, a previously defined pRb-interaction domain in cyclin D1 is dispensable for transformation in cyclin D1, but apparently not in the closely related cyclin D2. Instead, loss of a cyclin D1 domain just C-terminal the pRb-interaction domain may inactivate the protein. Using our knowledge of cyclin D1 function from these mutants and others, we hope to eventually create animal models of D1-dependent tumorigenesis useful for identifying suppressors of these tumors.

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INTRODUCTION

Breast cancer, like other cancers, results from the hyperactivity of growth-promoting oncoproteins and the loss of growth suppressing proteins (tumor suppressors). Many oncoproteins and several tumor suppressor proteins have recently been identified. Among the most commonly altered tumor suppressor proteins is the retinoblastoma protein, pRb. pRb function is lost in all retinoblastoma tumors, where it can lead to hereditary cancer, but is also involved in a variety of other tumors due to somatic inactivation. Reintroduction of the RB-1 cDNA into such cells inhibits their proliferation, supporting the role of pRb-inactivating mutations in formation of neoplastic cells (reviewed in Weinberg, 1991). This tumor-suppressive property of pRb is believed to result from pRb's ability to regulate progression through the cell cycle.

A central role for pRb in the control of cellular proliferation is also suggested by the observation that pRb is targeted by the oncoprotein products of several DNA tumor viruses (Whyte, et al., 1988; DeCaprio, et al., 1989; Egan, et al., 1989; Munger, et al., 1989; Dyson, et al., 1989). This interaction presumably serves to inactivate the growth-suppressive properties of pRb in infected or transformed cells. One mechanism by which oncoprotein-mediated inactivation may be achieved is through the dissociation of protein complexes between pRb and growth promoting molecules. For example, pRb has been reported to associate with the transcription factor E2F, which may be involved in the regulation of many genes required for DNA synthesis (Bagchi, et al., 1991; Bandara and LaThangue, 1991; Chellapan, et al., 1991; Chittenden, et al., 1991; Shirodkar, et al., 1992). The association of pRb with E2F may prevent the activation of these genes until the G1/S boundary, at which time the pRb/E2F complex dissociates, resulting in the release and activation of E2F. Because association of pRb with E2F seems to involve the same region of pRb (the "pocket") that is required for association with the viral oncoproteins, the binding of viral oncoproteins to pRb may release and activate E2F, resulting in the removal of a block to progression into S phase.

As is the case with a variety of human tumor types, some thirty percent of breast tumors show loss of pRb expression (Weinberg, 1991); however, other tumors have apparently wild-type pRb, and may have suffered alterations in one or another cellular proteins which interact with pRb. This may in turn lead to constitutive inactivation or circumvention of pRb function. A clue to the identity of such regulators of pRb is given by the fact that pRb is normally controlled by phosphorylation mediated by cyclin-dependent kinases (cdks; Lin, et al., 1991; Lees, et al., 1991). These cdks are controlled in turn by cyclins, regulatory subunits which lead to cyclic activity of their partner kinases. The activity of cyclin/cdk complexes is further regulated by positive and negative phosphorylation of the cdk subunit. In addition, several proteins have recently been identified that serve to stoichiometrically inhibit the function of cyclin/cdk complexes (reviewed in Morgan, 1995). Thus, these cyclin-dependent kinase inhibitors, or CKIs, together with cdk-modifying enzymes and cyclins represent potential targets for oncogenic mutations that may lead to deregulated cell cycle progression.

pRb is a critical target of cdks acting in G1, since hyperphosphorylation of pRb dissociates transcriptionally-repressive pRb/E2F complexes and allows cell cycle progression. pRb's phosphorylation occurs initially in late G1 phase, thus cyclins and cdks active in this period of the cell cycle are of most interest in regulation of pRb function. Three classes of cyclins expressed in G1 (C, D and E) have been identified and a variety of experiments have now shown that D and E type cyclins can regulate pRb phosphorylation and cell cycle progression. For example, by developing a cell culture assay for pRb function, we have shown that three cyclins, E, A and D1, can prevent pRb-mediated growth suppression when overexpressed in tumor cells (Hinds, et al., 1992). Cyclins E and A, which are expressed in late G1 and S phase respectively, appear to function by directly promoting the

phosphorylation of pRb in these cells. Cyclin D1, however, does not promote phosphorylation in our hands and thus appears to function differently from the A and E type cyclins, perhaps by reducing the amount of pRb present in cotransfected cells (Hinds, et al., 1992). Nevertheless, others have suggested that the D-type cyclins can activate kinase subunits related to those activated by cyclins A and E, perhaps resulting in the phosphorylation of a least a subset of pRb residues (Ewen, et al., 1993; Kato, et al., 1993). Further, we and others have shown that D-type cyclins can physically associate with pRb in a manner analogous to the viral oncoproteins (Ewen, et al., 1993; Kato, et al., 1993; Dowdy, et al., 1993).

Importantly, cyclin D1 has been shown to be overexpressed in some thirty percent of breast tumors (Lammie, et al., 1991; Schuuring, et al., 1992; Buckley, et al., 1993; Keyomarsi and Pardee, 1993), as well as in cancers of the parathyroid, blood and squamous epithelium (Motokura, et al., 1991; Rosenberg, et al., 1991a,b; Withers, et al., 1991). Thus, deregulated D-type cyclin expression may be oncogenic, leading to aberrant cellular proliferation perhaps by interfering with the function of pRb. We have recently shown that cyclin D1 can indeed act as an oncogene, cooperating to transform cultured cells in cooperation with a mutant adenovirus E1A oncoprotein which has lost the wild-type capacity to bind and inactivate pRb (Hinds, et al., 1994). This function of cyclin D1 requires complex formation with cdks, and may reflect a functional requirement for activation of this enzymatic activity.

Intriguingly, we have found that a mutant cyclin D1 protein which cannot bind to cdks not only fails to transform primary cells, but is dominant over the wild-type protein in this capacity. Thus, cointroduction of mutant and wild-type cyclin D1 genes leads to no increase in transformation frequency, suggesting a dominant-negative function of the mutant protein. This mutant protein can be expressed in conjunction with wild-type E1A, however, and is thus not itself lethal to cells in the absence of overexpressed cyclin D1. More detailed understanding of the mechanisms behind the inhibition of cyclin function could lead to antiproliferative products using existing technologies and may be specific to those cells overexpressing certain cyclins, leaving normal proliferating cells relatively unaffected. The properties of D-type cyclins already uncovered in our prior research and those to be elucidated by the experiments described below provide an excellent opportunity for clinical intervention in aberrant cell cycle control, and could thus provide an important adjunct to the pharmaceutical treatment of human cancers. In addition, we seek to identify other upstream regulators or downstream targets of pRb which can inactivate pRb upon overexpression. Such candidate oncoproteins may be operative in the significant fraction of tumors which do not show direct inactivation of pRb. To address these issues, the following specific aims are proposed:

1) Use mutant pRb proteins that have been characterized for function as growth suppressors and substrates for the pRb kinase to determine if the association of D-type cyclins with pRb is required for pRb function or phosphorylation or both.

2) Identify a kinase activity precipitable with antibodies specific to a tagged D-type cyclin and use this functional assay to probe the effect of pRb binding on D-type cyclin function.

3) Introduce a dominant-negative D-type cyclin into cells transformed by wild-type D-type cyclins and control cells in effort to specifically prevent the proliferation of the transformed cells.

4) Identify other substances, e.g. antisense oligonucleotides or interfering peptides, which could cause cessation of proliferation of cyclin D-transformed cells in vitro and in vivo.

5) Use cell lines temperature-sensitive for growth due to the expression of temperature-sensitive pRb to identify upstream inactivators and downstream targets of pRb.

RESULTS

Specific aims 1 and 2: Mapping of pRb and cyclin D1 domains required for transformation.

Although it is clear that cyclin D1 can function as an oncogene and does so at least in part by interfering with the pRb protein, the exact mechanism by which this is achieved is unclear. For example, cyclin D1 may simply activate its cognate kinase partners, cdk4 and cdk6, to directly phosphorylate pRb. Alternatively, excess cyclin D1/cdk complexes may compete with or lead to inactivation of any of a variety of CKIs now known to regulate cdk activity. Finally, although the absence of pRb seems to negate the need for cyclin D1 in tumor cells (Lukas, et al., 1995; Parry, et al., 1995), cyclin D1/cdk complexes may have other cellular targets. Examples of such targets are the pRb homologues p107 and p130 (Beijersbergen, et al., 1995). These proteins are homologous to pRb in the region used to associate with viral oncoproteins. Indeed, these proteins were first identified due to their association with E1A. It is now clear that p107 and p130 associate with transcription factors capable of binding to the E2F DNA site (Shirodkar, et al., 1992; Devoto, et al., 1992; Cao, et al., 1992; Cobrinik, et al., 1993), thus the homology to pRb is functional as well as physical. However, the E2F proteins that associate with pRb do not appear to interact with p107 or p130 in cells (J. Lees, personal communication). It is therefore likely that pRb, p107 and p130 are members of a family of proteins which regulate the function of a family of E2F transcription factors whose roles may or may not overlap in cell cycle control. Deregulated phosphorylation of these targets and pRb together may be more profoundly oncogenic than deletion of pRb alone, consistent with the high incidence of cyclin D1 overexpression in certain tumor subsets, such as is the case in breast cancer. We are attempting to identify the functional regions of cyclin D1 operative in transformation and correlate these with an induction of pRb phosphorylation. In addition, we will identify the minimal elements required to produce a dominant-negative version of cyclin D1 that may be useful in interfering with cyclin D1-mediated tumorigenesis.

Phosphorylation and transformation properties of mutant cyclin D1 proteins.

To define the regions of cyclin D1 needed for transformation, we have initiated a series of experiments using mutant cyclin D1 proteins. First, we are examining the ability of these cyclin D1 mutants to transform primary BRK cells in cooperation with the pm928 allele of E1A. We have focused in particular on the N-terminus of cyclin D1 at the beginning of these experiments, since this region contains a known pRb-interaction domain. Previous work had shown that a point mutation in this domain does not alter cyclin D1's ability to transform cells or inactivate pRb in human tumor cells (Hinds, et al., 1994). Interestingly, this differs from the reported inability of a cyclin D2 mutant lacking the pRb binding domain to phosphorylate and inactivate pRb (Ewen, et al., 1993). However, the cyclin D2 mutant used had a deletion of this domain, rather than a point mutation. We thus sought to elucidate any potential differences between these D-type cyclins. We constructed precise deletions of the pRb-binding, LxCxE domain in both cyclin D1 and cyclin D2 (Δ5 and Δ3, respectively) and tested both these mutants and the parent, wild-type alleles in transformation assays. As previously found, cyclin D1 can cooperate with 928 in BRK transformation. The Δ5 mutant of cyclin D1 was found to have equal or greater activity in these assays, consistent with previous results using point-mutated cyclin D1. Cyclin D2 cooperates at least as well as cyclin D1 in these assays, giving rise to foci with nearly fifty percent the frequency of wild-type E1A. Interestingly, preliminary results suggest that Δ3 cannot transform BRK cells efficiently, suggesting that cyclin D2, but not cyclin D1, depends on an interaction with the pRb pocket for transformation.

These mutant D cyclins and others have been used in parallel assays to determine their ability to promote pRb phosphorylation in transfected SAOS-2 cells. In this assay, cyclin D1 and cdk4 cotransfected with pRb leads to phosphorylation of pRb as judged by immunoblot of SDS-PAGE-treated cell extracts. The cyclin D1Δ5 mutant is wholly able to induce pRb phosphorylation in this assay, indicating that this interaction domain is dispensable for this activity in overexpression systems. Interestingly, the cyclin D2Δ5 mutant appears to only weakly induce pRb phosphorylation, again suggesting a dependence of cyclin D2 on this domain for pRb inactivation. Further, these results suggest a positive correlation between the ability of D-cyclins to stimulate pRb phosphorylation and transformation. To test this correlation further, we have employed two additional cyclin D1 mutant proteins. These mutants, Δ15 and Δ35, lack five amino acids beginning at residue 15 and 35, respectively, and are unable to induce pRb phosphorylation in SAOS-2 cells, although they are produced in levels equal to the wild-type cyclin D1 protein. The ability of these D1 cyclin mutants to transform BRK cells is being tested. Regardless of the outcome, we also plan to determine why these mutants fail to phosphorylate pRb. To do so, we will test the ability of these mutant cyclin D1 proteins to bind to pRb and to cdk4 and to enter into complexes with the p21 CKI. If these mutants do bind to cdk4, we will test their ability to activate this enzyme by performing kinase assays in vitro using a pRb C-terminal domain protein as substrate. In combination, these experiments should reveal properties of cyclin D1 needed for both pRb phosphorylation and transformation.

The set of reagents described above is also of utility in analyzing a second mechanism by which cyclin D1 may antagonize pRb. As stated above, the introduction of pRb, cyclin D1 and cdk4 into SAOS-2 cells leads to phosphorylation of pRb. However, if cdk4 is omitted, pRb is not phosphorylated but the level of pRb is significantly lowered. By using co-introduced reporter constructs driven by promoters equivalent to those used for pRb, we have determined that this reduction of pRb level is post-transcriptional in nature. Intriguingly, we have also observed that this pRb reduction is not caused by cointroduction of cyclin D2, once again demonstrating a significant difference between the otherwise highly related cyclins D1 and D2. Using mutants of pRb, our preliminary data suggests that integrity of the pRb "pocket", or oncoprotein binding domain, is not required for this effect. Further, the N-terminal, pRb-pocket binding region of cyclin D1 is dispensable for pRb reduction, and there appears to be no requirement for a functional cyclin box in this assay. Thus, reduction of pRb levels by cyclin D1 appears to be a unique property of cyclin D1 mediated by elements of both pRb and cyclin D1 that differ from those conventionally thought to participate in phosphorylation. Analysis of other cyclin D1 and pRb mutants is ongoing in effort to define the biochemical nature of this interaction.

A third series of experiments designed to test for pRb-independent pathways of cyclin D1-mediated transformation is underway. Because cyclin D1 can interact with and perhaps phosphorylate pRb-related proteins (Beijersbergen, et al., 1995), overexpression of this cyclin may activate growth-regulating pathways other than those controlled directly by pRb. As an example, it has been observed that overexpression of cyclin D1 in the mouse breast leads to hyperplasia within two weeks of birth (Wang, et al., 1994). However, chimaeric mice nullizygous for pRb do not show overt signs of breast hyperplasia (Hinds, 1995), possibly indicating additional roles for cyclin D1 in breast cell growth. We are using mouse fibroblasts derived from pRb-nullizygous embryos to test for roles of cyclin D1 in transformation that are independent of pRb. First, we have used wild-type E1A and ras to demonstrate that these cells can be transformed by this combination of plasmids (but not be either plasmid alone). Second, transfection of these cells with ras and the pm928 E1A mutant, defective primarily in pRb association, yields a high number of foci. Thus, genetic elimination of pRb compensates for the defect in this E1A protein. However, when pRb-minus fibroblasts were transfected with an E1A mutant lacking its LxCxE sequences entirely, and thus incapable of associating with pRb, p107 and p130, the efficiency of focus formation dropped to well under forty percent. This implies that CR2 functions other than pRb binding are needed for efficient transformation by E1A. We are now attempting to complement the defect in this multiply-defective E1A protein with cyclin D1 and mutant variants. If focus formation is enhanced, we will have clear evidence of a non-pRb-dependent effect of cyclin D1 in transformation.

Specific Aims 3 and 4: Suppression of D1-dependent tumor cell growth

We wish to establish transformed cells lines expressing various mutant alleles of cyclin D1 as described above prior to investigating the ability of dominant negative variants of cyclin D1 to suppress growth. At that time, we will test both BRK derivatives and breast tumor cell lines such as MDA-MB-134, 175, 330 and 453, all of which overexpress cyclin D1. As controls, we will use E1A-plus-ras transformed BRK cells and breast tumor lines such as MDA-MD-468, which has lost pRb expression and does not highly express cyclin D1.

An important goal of these aims is to move such growth suppression experiments in vivo. To do this, we are creating mice that should develop D1-dependent tumors. In collaboration with Dr. Terry van Dyke at UNC Chapel Hill we have created transgenic mice expressing cyclin D1 under the LPV enhancer. This construct allows cyclin D1 expression in the choroid plexus, a tissue in which Dr. van Dyke has already established a tumor model. Here, variants of SV40 Large T Antigen have been expressed that fail to bind to pRb and fail to cause tumors. We hope to complement this defect by mating the T mutant and cyclin D1 mice. At present, the LPV-D1 transgenics appear to express cyclin D1 in the choroid plexus, and at low penetrance develop hydrocephaly, indicating a potential choroid plexus defect. The cause of this is under investigating. In any case, these mice appear suitable for establishing a D1-dependent tumor model. Coupled with transgenic mice that will be produced expressing cyclin D1 in the breast (see below, Discussion), we believe these model systems will allow in vivo testing of potential anti-cyclin D1 reagents.

Specific Aim 5: Establishment of cell lines expressing temperature-sensitive pRb.

A linker-insertion mutation in pRb at codon 668, resulting in the insertion of four amino acids, produces a pRb protein (XX668) that fails to induce the G1 growth arrest in SAOS-2 cells that is characterized by the "flat cell" phenotype. However, when the construct encoding this mutant protein is introduced into SAOS-2 cells incubated at 32.5°C, the activity of the mutant pRb is indistinguishable from that of the wild-type protein. This temperature-sensitive effect should allow the establishment of cell lines that proliferate at 37°C, but uniformly undergo growth arrest in G1 at 32.5°C.

Preliminary experiments suggest that such cell lines can be created by transfection of XX668 into SAOS-2 cells. These cloned cell lines proliferate similar to parental cells at 37°C, but undergo growth arrest and phenotypic change at 32.5°C. Initial characterization of the cells at both temperatures indicates that the level of pRb doesn't change dramatically, but the protein is only able to bind substrates at 32.5°C. In addition, no significant change in the expression of G1 cyclins has been noted. Despite this promising beginning, cell lines expressing XX668 at 37°C appear to be rather unstable, in that they lose pRb expression with continued passage, perhaps due to a weak but significant partial activity of the pRb. We are now attempting to create derivatives of XX668 that will be more tolerated by SAOS-2 cells, and we are also in the process of introducing XX668 and derivatives into DU145 prostate adenocarcinoma and MDA-MB-468 breast carcinoma cell lines, both of which lack normal pRb expression.

Our previous (unpublished) work using pRb mutants lacking the nuclear localization signal demonstrated that the loss of this targeting sequence produces a protein that localizes predominantly to the cytoplasm, yet maintains some nuclear localization. Further, the fraction of pRb that localizes to the nucleus appears functional, since it resists detergent treatment and causes growth arrest in the cells containing a significant nuclear fraction. This phenotype is consistent with the location of the NLS mutation at residue 860-876, a region that has not been implicated in any of the protein-protein associations required for pRb function. Curiously, when the NLS mutation is crossed with a tumor-derived inactivating mutation such as C to F at residue 706, the resultant doubly mutated protein is

exclusively cytoplasmic and cannot induce growth arrest. We believe this indicates that the "pocket" of pRb is responsible for the "residual" nuclear localization of the protein, perhaps by association with nuclear structures upon their synthesis and transport to the nucleus.

We have introduced the NLS-minus mutation into the XX668 tsRb background in anticipation of creating a pRb protein that will be cytoplasmic and nonfunctional at 37°C, but will regain pocket function and thus partial nuclear localization at 32.5°C. Such a mutant may be better tolerated by SAOS-2 cells since very little of the protein will be nuclear at the nonpermissive temperature. Cell lines derived from the multiple mutant, or stable versions of breast tumor lines expressing the original tspRb, should be valuable in studying the molecular events that occur upon pRb activation as well as those that result in renewed proliferation after pRb is inactivated.

CONCLUSIONS

Our ongoing work characterizing the method of action of cyclin D1 as an oncogene will be useful in identifying the functions of cyclin D1 that may serve as targets for anti-tumor approaches. Preliminarily, it seems that cyclin D1 differs from cyclin D2 in its biochemical effects on pRb in overexpression systems. These differences may explain the common observation of cyclin D1 as a human oncogene, in contrast to the rare incidence of cyclin D2 overexpression. Such differences are what we hope to exploit to reverse the aberrant proliferation resulting from cyclin D1 overexpression.

In a similar fashion, we believe that cyclin D1 overexpression may have consequences other than, or in addition to, direct inactivation of pRb. The different phenotypes associated with cyclin D1 overexpression as compared to pRb deletion support this notion. Our attempts to address this issue in pRb-minus fibroblasts show some promise, but need to be extended in animal models. To address this, we have very recently initiated a collaboration with Tyler Jacks. Dr. Jacks will provide embryonic stem cells lacking the pRb protein due to two independent gene targeting events. We will introduce breast-specific cyclin D1 constructs into these cells and control, normal cells to produce transgenics. The resultant cells should produce chimaeric mice with or without pRb and expressing cyclin D1 at high level in the breast. Based on published results, cyclin D1 overexpression should lead to hyperplasia and eventually tumor formation in the norm background. If this is also observed in pRb-minus breast tissue overexpressing cyclin D1, there are likely to be other targets of cyclin D1 relevant to breast tumor formation. A lack of hyperproliferation in this case would suggest that cyclin D1 cannot contribute to tumor formation in the absence of pRb.

Together, we hope that these experiments will extend our understanding of the role of cyclin D1 (and pRb) in breast cancer. As these experiments yield data on the functions of cyclin D1 regions in transformation, we will proceed with experiments designed to suppress D1-dependent tumor cell growth (specific aims 3 and 4). Precise knowledge of the functions of cyclin D1 in tumorigenesis may well demonstrate a specificity of overexpressed cyclin D1 that can be targeted in tumor cells, leaving normal cells unscathed.

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